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Heavy Metal Pollutome and Microbial Resistome Reciprocal Interaction and Its Impact on Human and Animal Matrices

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Additional information is available at the end of the chapter

Abstract

The chapter aims to reveal the complex relationships between antibiotic resistance in bacteria and heavy metal pollution at the human/animal interface. The antibiotic resistance is a continuously growing threat for both people and animals. Animals could represent a source for zoonotic microbial contamination of humans as subject for consumption and also as contacts (companion, sports, zoo animals, etc.). Antimicrobial treatments in animals, if uncontrolled or injudicious, could raise antibiotic-resistant strains to be transferred to humans where they can cause even more severe diseases. Moreover, the environment has its own microbiome, including some nonpathogenic but antibiotic-resistant species. Human industrial activities are carried out in certain environments, with particular microbiomes and also where animals bearing antibiotic-resistant bacteria are present. Thus, the degree of pollution with heavy metals, as part of the global pollutants to the environment, could impact on the bacteria and their resistome with severe consequences for inhabitants of the area.

Keywords: antibiotic resistance, heavy metals, zoonotic bacteria, pollution

1. Introduction

Heavy metal pollution represents a significant part of general pollution subsequent to human activities. Lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are some of the most widespread pollutants, which pose serious threats to human, farmed animal, and wildlife

health [1, 2, 4–8]. These pollutants interact with the genome of the exposed individuals and interfere with the development/survival of species. Humans are positioned at the crossroad of several exposure routes, such as (a) habitat exposure [10]; (b) consumer exposure; and (c) work place exposure [9]. Heavy metals, as pollutants in various microbial habitats, could change the plasmids, inducing antibiotic resistance. In spite of numerous researches dealing with heavy metal pollution and its influence on humans, less of the studies concern the impact of heavy metal pollution on farmed and especially wild animals and their bacteriome, beyond the effects on their productions: meat, milk, or eggs.

Similar to heavy metal pollution, termed "pollutome" in this chapter as in Refs. [11, 12], resistance to antibiotics stands lately for an issue of broad community concern [13–15]. Biased and exaggerated antibiotic treatments, applied with no clinical reasoning, in both humans and animals, have subjected the microbial community to a strong selective pressure that led to adaptation and appearance of resistance plasmids looked at as "resistome." Recent investigations suggested the intervention of various factors changing the bacterial metabolism and subsequently, the ultrastructure and, eventually, the resistance to antibiotics. The "resistome" is continuously increasing, due to the further replacement of older generation antibiotics with newer ones and concurrent influence of other environmental factors. Due to their continuously increasing numbers, exploration was dedicated to the transfer of multidrug-resistant (MDR) bacteria from farmed animals/food products to humans [16, 17].

The spread of pathogens in the environment subsequent to human activities may cause diseases in humans, livestock, and wildlife [18–20]. The intensive technologies for food animal raising still use the antimicrobial agents at a large scale, in order to treat the infectious diseases in animals and to reduce the mortality and economic impact of diseases. Abusive use as well as misuse of antibiotics induced increased percentages of resistant bacteria. Infections by resistant and MDR bacteria endanger the human population and animals exposing those to sometimes uncontrollable threats [20, 21, 22].

A recent study has shown that in soil samples, in particular in those where the phenomenon of crossing species-specific barriers is present, the occurrence of heavy metals is associated with elevated antibiotic resistance. It was also indicated that the geo-chemical metal conditions innately influence the potential for antibiotic resistance in the soil [23]. The importance of understanding the soil resistome in the preservation of antibiotics for the treatment of infections was highlighted [24]. The elevated frequency of both metal and antibiotic tolerances in bacterioplankton from metal contaminated sites has been identified by [25]. The associations between the types and levels of metal contamination and specific patterns of antibiotic resistance indicated several mechanisms that underlie this coselection process, including coresistance and cross-resistance [2, 26, 27]. Therefore, it was suggested that metal contamination represents a selective pressure with both environmental and clinical importance that potentially contributes in maintaining and spreading antibiotic resistance factors [2] and also increasing the risk for both humans and animals in specific areas (**Figure 1**).

Due to peculiarities of multiplication and physiological traits of bacteria such as rapid growth, this antimicrobial resistance could spread to naive microbial populations. Identification of the relationship that exists between heavy metal pollutants, which by themselves negatively affect
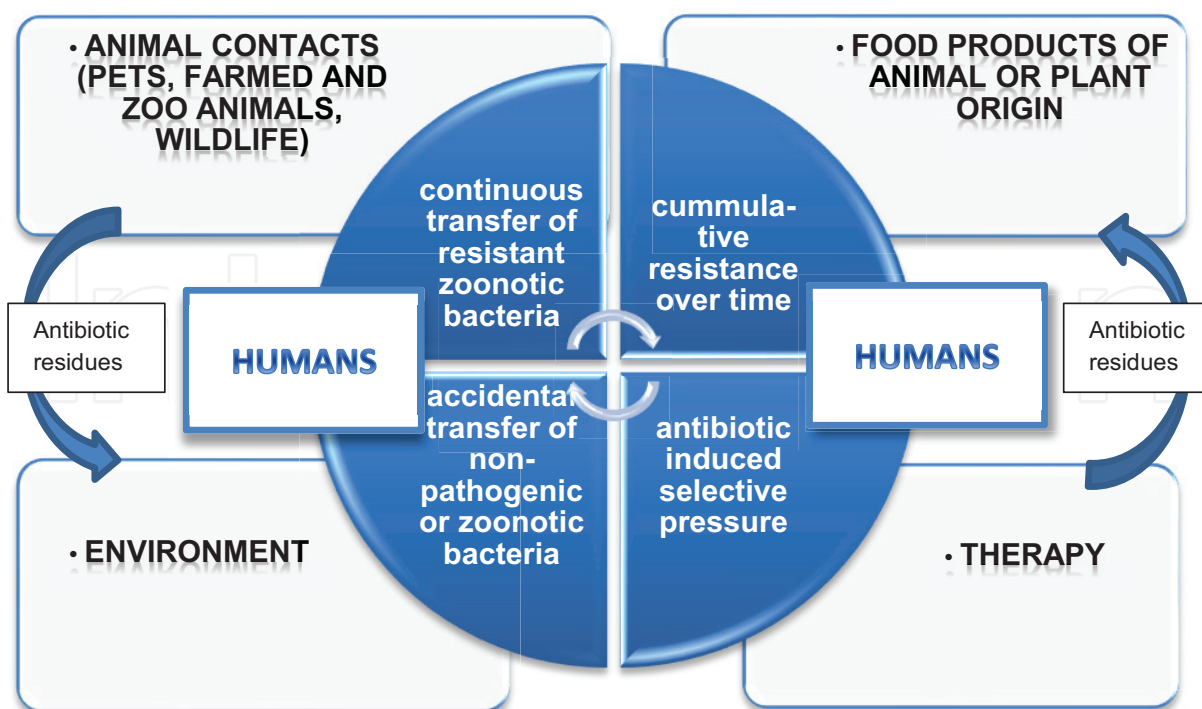
Figure 1. Main sources for human exposure to antibiotic-resistant bacteria.

human and animal health, and pathogenic or potentially pathogenic bacteria showing various degrees (MDR or HARS) of antimicrobial resistance could substantially improve the health care programs and allow the identification of crucial points where implementing a better control strategy is of utmost importance. Thus, not only the human and animal welfare will be improved but a substantial progress toward environment and ecosystem protection could be accomplished.

2. Heavy metal pollutome and its impact on health

The most common heavy metal pollutants are arsenic, cadmium, chromium, copper, nickel, lead, and mercury; because of the environmental and health effects in human and animal ontogeny, lead, mercury, and cadmium are of the greatest concern.

Present naturally or secondary from anthropogenic sources in the environment, the heavy metals are mostly nonessential to humans and other organisms and only some of them (e.g. copper, selenium, zinc, etc.) are essential in small quantities for the metabolism of the living organisms [1]. The main characteristic of heavy metals consists of their toxicity at low concentrations. Heavy metals enter the organism by ingestion via food, drinking water and, in some particular cases, soil, and by inhalation via air.

The lifetime of heavy metals in the environment can vary, but some of them can persist in soils for tens or even hundreds of years. There are no doubts that the soil and water ecosystems are the most impacted by heavy metal contamination.

Even though the most important disasters caused by heavy metals have been due to the massive contamination of water and soil and secondary contamination of food (Minamata, Japan, 1932; Sandoz, Germany, 1986; Coto de Donana, Spain, 1998), the historical (persistent) pollution of the soil with heavy metals still remains a problem of global concern.

In this framework, Romania represents an important example. Here, hot spots for heavy metal contamination of the environment are operational and former mining sites (extractive industry), particularly smelters, contribute to air, soil, and water pollution. In these areas, a number of studies have shown that the soils are polluted by heavy metals, especially in the proximity of metallurgical smelters and tailing dams [4], also representing a significant contamination source for water and vegetation [4, 8, 28]. Frequently, heavy metals are dispersed to long distances affecting broad land surfaces, which include agricultural land and forest funds. The increase of the natural soil acidity and soil contamination with heavy metals contributed to soil base depletion, microbiological disturbance, organic matter degradation, soil structure deterioration, and others [7, 29, 30].

Generally speaking, soil contamination is an actual universal problem. In this frame, pollution of soils by heavy metals in urban areas is not an exception and is mostly due to former industrial activities and traffic. The redistribution of heavy metals in urban areas is known to be strongly correlated with historical pollution, the best example in this regard being the lead contamination. Recent evidence [31, 32] indicates that urban soils have been moderately to highly polluted by Cd, Zn, Cu, and Pb originating in current industrial activities (e.g. steel industry). Also, topsoils near the smelting plants of molybdenum concentrate have had moderately to extremely high contamination levels for Mo and Pb. When it comes to urban public places, several studies [31–33] have showed that a site polluted by metals (e.g. Pb, Zn, Ni, As, and Mo) could pose a high noncarcinogenic health risk, or, on the contrary, the noncarcinogenic and carcinogenic risks could be insignificant or within acceptable limits, depending on the contamination degree and land use. On the other hand, in children, the most sensitive population, the risk assessment showed that they are at high multielemental noncarcinogenic risk [32].

Another source of soil pollution and transfer to different environmental components (water, plants, and animals) from urban areas is represented by waste materials. All types of municipal solid waste and sewage sludge contain many heavy metals with a significant impact on the environment, primarily increasing their levels in soil. Such wastes and sewage sludge added to agricultural and other soils lead to higher heavy metal content and the availability of heavy metals for transfer into crop plants with implicit risk to human health. It has to be mentioned that the composting process reduces the metal availability and the impact on the ecosystems [34, 35].

As it has been indicated, heavy metal pollution had a significant impact on the environment and ecosystems, causing the emergence of negative effects in all forms of life. Lead, cadmium, mercury, and arsenic are toxic for plants, wildlife, experimental animals, and humans. Bioavailability and bioaccumulation are key factors in their toxicity in live organisms and that is why most diseases associated with heavy metal pollution are the chronic ones, sometimes systemic, and generally are results of long-term/low level of exposure [1, 10, 35]. Due to their transfer chain between different matrices, bioavailability, and accumulation in live organisms, heavy metals are potentially harmful at some level of exposure and absorption, this property conducting to severe disorders [1, 5, 34–36].

All metal pollutants can reach the aquatic environments and the concentrations of heavy metals in trophic chain (e.g. fish) can be much higher than those found in aquatic environment or in sediments [37]. Even without major contamination sources, heavy metals are found in sediments (deposits) due to contamination and transportation from the river basins, higher concentrations being related to the lower velocity of the water flow. Sediment contamination affects primarily benthonic organisms but, indirectly, aquatic beings as well, due to their higher trophic level or by heavy metal remobilization to the overlying water [38]. The bioaccumulation of some metals (Hg, Cd, Cr, Cu, and Pb) in two species of fresh water fish from Yonki Reservoir has been reported, even though the concentration has been safe for human health [39]. As for the marine environment contaminated by several heavy metals (Cu, Zn, and Cr), some concerns were noticed, particularly in terms of safety for human consumption of aquatic animals captured from different areas in North-Eastern Mediterranean Sea [37].

Heavy metal pollution is noteworthy because of the risks it poses to human health. Lead, cadmium, mercury, and arsenic affect in different proportions, and mostly in an irreversible way, the central and peripheral nervous systems and cardiovascular, renal, reproductive, hematopoietic, and immune systems. These effects have been extensively studied. The concern on the frequency and magnitude of health effects is very high, as long as the body burden of cadmium, mercury, and lead depends mostly on the dietary intake of these elements.

Recent data indicate that adverse health effects of lead, cadmium, and mercury may occur at lower exposure levels than previously anticipated, especially for lead and methylmercury, which generate neurotoxic effects such as developmental delays, neurobehavioral dysfunction, attention deficit, hyperactivity disorder in susceptible population, and a decrease of intelligence quotient (IQ) in children. One still incompletely answered question is "what are the interactions when an individual is exposed to the combination of lead and methylmercury?" [40]. Moreover, a very recent study emphasizes the economic benefits of methylmercury exposure control in Europe [3]. The renal tubular dysfunction, a critical effect of long-term exposure to cadmium, is irreversible. Long-term drinking-water arsenic exposure is mainly related to increased risks of skin and other cancers, as well as skin lesions [40]. Thus, growing environmental pollution by heavy metals probably contributes to the enhanced incidence of allergic diseases and cancers in urban populations.

In the last years, several studies have been focusing on the reproductive and immunotoxic effects of heavy metals, looking for the mechanisms of these effects with severe consequences on human and animal health. In contrast with the numerous studies that have observed reproductive effects in occupationally exposed humans and experimental animals with high exposure, the studies concerning the effects of low levels of these metals on male reproductive outcomes are limited. Even so, the evidence for the effects of low exposure was the strongest for cadmium, lead, and mercury and less certain for arsenic [41].

There is proof that heavy metals influence the immune response of the body and most of the studies were focused on lead and cadmium because of the number of exposed individuals worldwide. For example, [42] was among the first authors to publish on lead, cadmium, and methylmercury immune toxicity in experimental animals, demonstrating the dose-response relationship of lymphocytes' memory to antigen. A very important observation refers to subclinical

amounts of the administered metal, which could affect the T lymphocyte when the secondary immune response is altered. In copper smelters, a lower production of IgA and IgG can be detected in targeted subjects, predisposing them to infections and cancers [43].

The higher intensity of exposure in recent studies on the work-related environment [9] suggests that occupational exposure to lead may disrupt the immune response and diminish immune prevention in exposed individuals.

Related to mercury, there is evidence that both inorganic and organic forms of it cause immune suppression and induction of autoimmunity. A study was conducted [44] to monitor the effect of inorganic mercury in an autoimmune heart disease model induced by infection with Coxsackievirus B3 in mice. The exposure to inorganic mercury before Coxsackievirus B3 infection functioned as an aggravating factor for the severity of the cardiac pathology induced by a macrophage infiltrate and mixed cytokine response in the heart.

More recently [45], it has been shown that lead up to 5.0 µg/dL affected the immune competence against pathogens, depending on bacterial species (*Escherichia coli* or *Salmonella typhimurium*), suggesting that Toll-like receptors, TLR4, were targets for the lead effect.

3. Antimicrobial resistance: patterns of human-animal-environment connections

At broad public and scientific community level, nationally and internationally, the expanding problem of antimicrobial resistance has been identified and tackled for several years, since antimicrobial resistance transpasses frontiers. The factors that favor bacterial resistance were partly investigated. Nevertheless, the therapy of bacterial infections and transmissible diseases still relies on antibiotic use and it is mostly being done without a previous testing of the resistance of the agent to various antimicrobials, regardless of environmental variables. Similarly, heavy metal pollution and preservation of ecosystems represented a central point of numerous national and international organisms' interest in developing strategies to ensure health preservation and drug production, marketing and use worldwide.

The appearance and expansion of antimicrobial resistance [19, 46] have become lately one of the main public health worldwide concerns. The detection, improvement, and placement on market of compounds with killing or growth-inhibiting effects on bacteria and other microorganisms, modernized the treatment of infectious diseases, helping with a dramatic diminishment of morbidity and mortality rates in humans and animals [14] and have substantially contributed to population health improvements. Antibiotics belong to the group of these compounds.

Nonetheless, disease-causing organisms have an outstanding ability to adapt, markedly to acquire and transmit antimicrobial resistance [47, 48]. Natural ecosystems, also including human gut, contain a large number of elements that can confer resistance to antimicrobials [46].

The antibiotic resistome concept indicates a dynamic process as a cause of resistance. This process involves microbial interactions in multiple environments, taking place ahead of the so-called antibiotic-era [26, 27].

The fact that resistant microorganisms can explore a wide range of potential niches and acquire optimal adaptations for life in alternative hosts is threatening human or animal health, by amplifying the capacity of bacteria to acquire new virulence and resistance determinants, meanwhile adapting to the habitat. Since the variability of these resistance determinants and their expression in different hosts are broader in nature than in human and animal pathogens, bottlenecks conditioning the transfer, spread, and stability of antibiotic-resistant genes must exist [49]. Moreover, the abusive and uncontrolled use of antimicrobial agents in both human and veterinary medicine facilitates the continuously increasing numbers of resistant organisms. These organisms have the capability to "commute" from animals to humans and back [16, 17] (**Figure 2**).

Considering that the origin of antibiotic resistance is the environmental microbiota [46], recent studies indicated that urban wastewater treatment plants are among the main sources of antibiotics' release into the environment and treated wastewater contains antibiotic-resistant bacteria or genes encoding virulence or antimicrobial resistance leading to the emergence of new pathogens [20, 50, 51]. Even though antimicrobial resistance from natural sources existed before antimicrobial agents were introduced into medical treatment [15], a strong connection can be established between the amounts of antimicrobials used and the increase of numbers of species resistant to them. Despite ongoing research to find new groups of drugs to combat
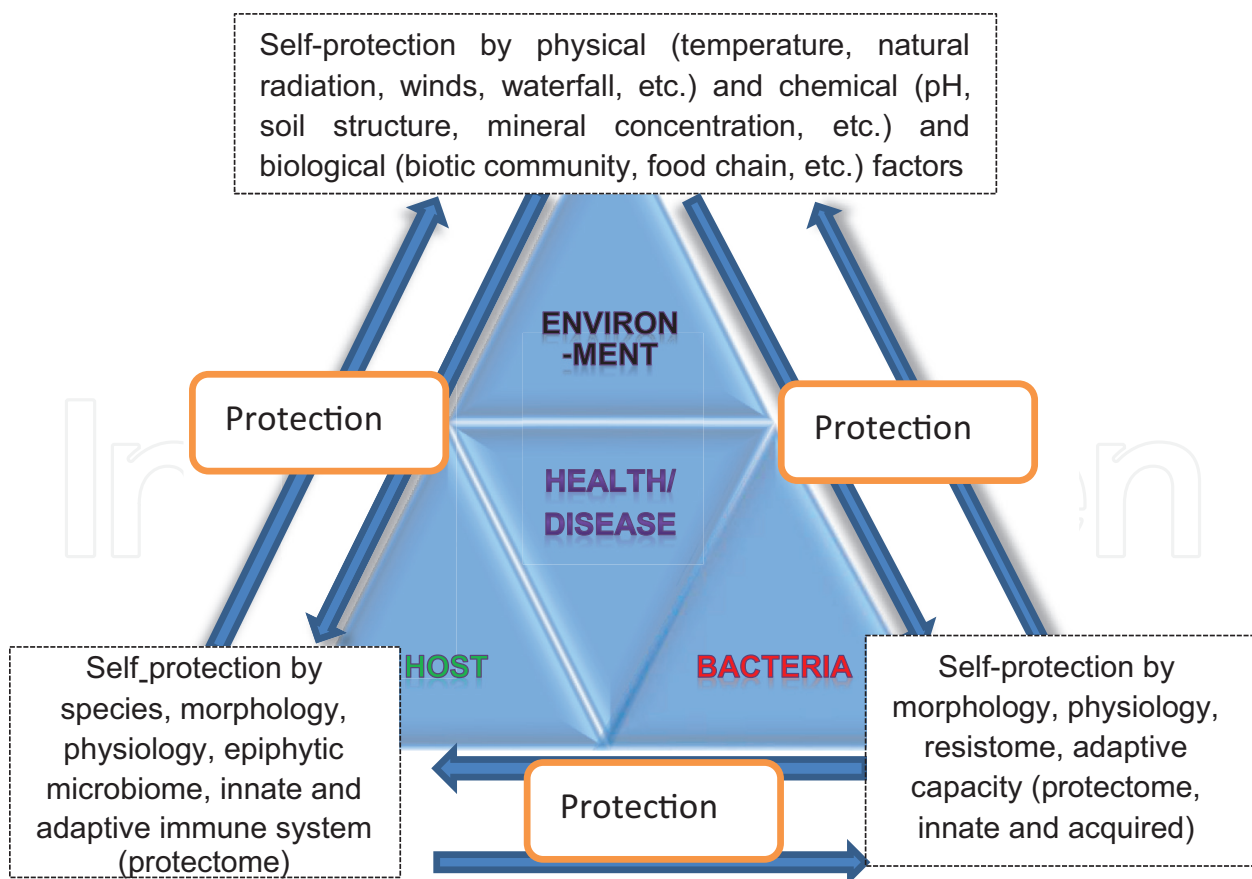

Figure 2. Multilevel regulating interactions within the epidemiological triangle that conditions health/disease outcome for the host.

resistant organisms, it is uncertain if and when such drugs will be available. Therefore, antimicrobial agents must be used prudently in order to limit the further emergence and spread of resistant germs [15].

Along with the manure used as fertilizer and spread onto agricultural fields, both residues of antibiotics and antibiotic-resistant bacteria may spread and pollute the environment [52]. Within this framework, the assessment of bacterial resistance in wild animals and game may be a valuable tool to monitor the environmental health and to manage emerging infectious diseases.

Several studies pointed out that fecal bacteria such as *E. coli* and enterococci of wild animals (wild boars, rabbits, and bison) [53] could be a reservoir of antimicrobial-resistant genes and frequently multiple resistant bacteria that could be transmitted to other animals or even to humans [54–56]. The authors suggest that wild animals can serve as sentinel populations for studying the origin and spreading of antibiotic resistance. Up to now, there are no studies to report on the antibiotic resistance in wildlife or humans from heavy metal–polluted areas.

In clinical practice, bacteria are being monitored for their resistance to antimicrobial drugs, which is an important predictor of treatment outcome. However, it is recognized that there is considerable heterogeneity in the metabolism of bacterial species and, in most of the cases, in their behavior toward the antibiotics. Methods for revealing the support of antimicrobial resistance, sometimes to multiple drugs, have developed from simple diffusion tests to gene detection, plasmid monitoring by molecular methods, and changes in their spectra following treatment by FTIR.

In considering the pollutome/resistome interaction, we identified the following bottlenecks: (a) heavy metal pollutants change the bacterial metabolism, inducing an increased resistance to antibiotics in the absence of antibiotic treatment; therefore, (b) in wild animals inhabiting polluted areas, antibiotic-resistant bacteria could be present without any previous contact with humans or domestic animals, but (c) the simultaneous activity of antibiotic treatment and pollution could induce the presence of an extremely resistant bacterial flora in humans, domestic animals, and wildlife contacts and pose a serious threat to their health/survival.

According to this, it is presumable that the exposure to heavy metals can emerge in severe infections in humans and animals, very difficult to treat because of the depletion of the immune system function and antibiotic resistance of the involved pathogen.

The identification of associations between genotype, resistance, and clinical outcome is obviously important under these circumstances. It could allow the prediction of likely outcomes in terms of aggravation of resistant bacteria–induced diseases and overall pollution, which is of critical importance to preventive strategies.

The identification of significant interactions between genetic loci and lifestyle risk factors could strengthen the evidence that heavy metal pollution factors are causally related to the emergence of MDR and could improve the understanding of the mechanisms through which these risk factors influence microbial diseases.

An effective preventive strategy requires close cooperation and consultation between all involved decision-making parties, especially at international level [13].

4. Influence of heavy metal pollution on antibiotic resistance

Since 1999, when the complex socioeconomic and behavioral backgrounds were first mentioned in developing countries as a cause of the escalating problem of antibiotic resistance, some studies went further investigating the environmental factors' contribution.

Secondary to pollution by heavy metals, the biology and chemistry of the soil can be influenced regarding the tolerance of bacteria to these toxic elements.

Some researches [57] have shown that plants can harbor different metal-resistant bacterial communities in their rhizosphere soils, *Pseudomonas* and *Arthrobacter* dominating the isolates tolerant to lead. The evaluation of the aerobic soil microbial population demonstrated the presence of considerable numbers of viable bacteria in soil samples from two long-term heavy metal highly contaminated sites [58].

A very recent study [59] on the relationship between the antibiotic and heavy metal tolerance of cultivable bacteria isolated from soils containing different levels of heavy metals showed a high rate of coresistance toward Hg and antibiotics among the Gram-negative isolates and toward Zn, Ni, Hg, and the beta-lactam antibiotics among the Gram-positive bacteria. Along with other factors influencing gene transfer between bacteria, the authors possibly relate higher percentage of isolates with multiple antibiotic resistance to the level of soil heavy metals and the population of soil bacteria. Also, the distribution of antibiotic-resistant genes in surface water (lakes) was mainly attributed to antibiotic and heavy metal coselection as a result of anthropic impact [60]. Other researchers [61] confirmed the links between cadmium accumulation and antibiotic resistance in *Salmonella enterica* serovar Typhi Ty2, because of the influx of heavy metal ions in the environment from where the infection is transmitted (e.g. sludge).

The information on the pollutome/resistome interaction that could intermediate the appearance of antibiotic-resistant strains in nonantibiotic "user" environments, such as wildlife microflora or nonpatogenic microbiota, in case of heavy metal pollution presence is scarce or lacking.

By taking into account various types of changes that could induce antimicrobial resistance, when relating the risk to genetic factors and gene-environment pollution interactions, specific pathways may be picked up, in which the effects of environmental risk factors may prove to be clearer, and therefore this approach may substantially increase the chance of finding relevant gene-environment interactions. In a broader perspective, detailed knowledge of the pollutome-resistome interaction is needed to prepare control plans for avoiding further spreading of such bacteria and eliminating the risk for humans and animals. In addition, genetic factors definitely influence the interaction between such bacteria and the susceptible hosts.

5. Interactions of heavy metal pollutome and bacteria in the Danube Delta Biosphere Natural Reserve: a case study

As a representative case study on the simultaneous presence of heavy metals (Pb, Cd, Hg, and As) and antibiotic-resistant bacteria in water system, toxicological and microbiological

pollution within the Danube Delta (**Figure 3**), a UNESCO Biosphere Reservation from Romania subjected to the broader European contamination transported by the Danube River, is presented. The research was carried out within the frame of a research project supported by the National Research Council of the Ministry of Education (PNII 61/2012).

5.1. Methods

Samples were collected from representative areas of the Danube Delta, but for a comprehensive case study, the area was assigned around the Sfântu Gheorghe settlement toward the mouth of the homonymous river branch. This area was certified and statistically supported by the similarities noticed with the level of the entire Danube Delta, and the same microbial species were isolated in both the research area and the Sfântu Gheorghe river branch.

The research has extended over a period of 3 years (2012–2015) during which samples of water and sediments were collected seasonally from the case study area, along with benthic and pelagic fish organs and microbiological swabs (Deltalab, Eurotubo). The water, sediment, and fish (perch, wels catfish, sander, pike, pontic shad, crucian carp, and common carp) samples were analyzed for heavy metals through atomic absorption spectrometry (AAS) method: Pb and Cd through electrothermal atomic absorption spectrometry (ET-AAS); As and Hg through hydride generation atomic absorption spectrometry (HG-AAS) supplemented with amalgamation and Cold Vapour Atomic Absorption (CVAA) method.

Figure 3. The Danube Delta within Romania and the case study area [62].

Isolation and identification protocols selected for targeted bacteria included initially bacterioscopy directly from the buffers (transport media) by Gram staining and cultivation on Tryptone Soya Agar (TSA, Biolab) medium and afterwards passages to peptone water and Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid) for isolation of *Vibrio* spp. (*V. cholera* and *V. parahaemolyticus*); CUM (Chromogenic UTI agar, Oxoid) medium for isolation of *E. coli*, enterococci, and coliforms; and BHI and Cetrimide + Nalidixic acid (AES Laboratoire) medium for isolation of *Pseudomonas aeruginosa*. Antibiograms on these isolates were performed by the Kirby-Bauer diffusion method, the disks containing 9 antibiotics (ciprofloxacin, penicillin, streptomycin, erythromycin, oxytetracycline, marbofloxacin, akamycin, enrofloxacin, and ampicillin). Inhibition diameters were read, and the presence of total resistance (R) or resistant colonies (partial resistance, CR) were recorded.

For studying the patterns regarding the spatial distribution of different parameters, we used advanced spatial analysis methods (GIS) that imply geostatistical interpolations (Kriging) with the use of natural barriers (water/land). To reveal seasonal patterns and multiannual tendencies in the measured variables, we used statistical analyses, starting from Pearson's correlation, t-test, or ANOVA, to advanced methods like principal component analysis (PCA) and canonical discriminant analysis (CDA).

5.2. Results and discussion

The environmental assessment held in the case study area showed an inconsistent detection of heavy metals, like Pb, Cd, and Hg, in the water samples, being influenced by the location and the sampling season. However, As was the only metal that was measured constantly, with perceptible, from small to medium, variations. Two metals with high toxicity, Cd and Hg, have exceeded the Romanian limits for surface water quality, according to the EU Directive for water quality, being in some cases even above the maximum class—V.

In sediments, the presence of metals was constant, rendering as follows: Pb > As > Cd > Hg, the last two (Cd and Hg) exceeding the Romanian quality limits for sediments.

By analyzing the seasonal variability of the quality of habitats during this research, the water values for Pb and As on one hand and those for Cd and Hg on the other had similar tendencies, but without a pattern being highlighted within the normal seasonal variation. However, the metal distribution in sediment was normal, with predictable seasonal variations that can be directly compared to the tendency observed in other reference studies. Therefore, with regard to the seasonal variation of heavy metals, it can be stated that the data indicate that sediments act as a reservoir of contaminants due to immobilization and remobilization processes that occur under different conditions depending on seasonal changes, confirming the study of heavy metals in sediments, by occasional, seasonal sampling, as a good method to describe the temporal variations in the status of aquatic systems studied.

Of the studied fish species, five out of six (carp, crucian carp, pike, sander, wels catfish, perch, excluding the pontic shad) showed average values of arsenic and mercury higher or equal in internal compared to external organs. In the case of cadmium, in all investigated species, the concentrations in the internal organs were significantly higher than those in the external

organs. As for the concentration of lead, it was in five out of six cases higher or equal in the external organs than in the internal ones, a situation opposite to the other metals.

The highest bioaccumulations of Pb and Cd were recorded in the nonpredatory species (carp, crucian carp, and carp, respectively). Predatory fish, holding a top position in the food chain, showed higher levels of Hg in both internal and external organs.

The assessment of the microbiome in the water and sediment indicated a high degree of pollution. A total of 197 bacterial strains (85, 43.14% in water and 112, 56.85% in sediment) were isolated. Most strains were isolated in July (a total of 66, 33.50%; 28, 42.42% in water; 38, 57.57% in sediment), followed by November (a total of 50, 25.38%, 19.38%, water; 31.62% in sediment) in March (a total of 44, 22.33%; 23, 52.27% in water; 21, 47.73% of sediment) and May (a total of 37, 18.78%; 15, 40.54% of water and 22, 59.45% of sediment). The highest prevalence of *E. coli* strains/coliforms was identified in July (9, 56.25%), while *Vibrio* spp. (*V. cholerae/V. fluvialis*, *V. alginolyticus/V. metschnikovii*, *V. mimicus/V. vulnificus*, *V. parahaemolyticus*) showed an increased incidence in November (7, 53.84%). The *Pseudomonas* spp. strains were isolated exclusively in November (2, 15.36%) and July (2, 12.5%), while *Proteus* spp. and *Enterococcus faecalis* were also present in large numbers, especially in July. In terms of staphylococcal strains, large seasonal variations were present in July (4, 25.00%).

Summing up the data, mainly coliform bacteria were isolated, more than half of the isolates being confirmed as *E. coli* from water samples. Next were ranked the species belonging to *Pseudomonas* genus, followed by *Vibrio* species, with an average to low level.

In the sediment, the frequency of positive samples with coliform bacteria was much higher, more than half of them being positive for *E. coli* as well. *Pseudomonas* spp. had a frequency close to the one in water, while *Vibrio* spp. had a lower frequency (except V. *cholerae*).

The microbial qualitative assessment of aquatic habitats indicated variability induced by seasonal fluctuations in the percentage of positive samples. Species of the genus *Vibrio* had similar variations in water and sediment, with a high peak in presence and diversity during late-2013 and mid-2014.

The frequency of coliforms was higher in sediment samples than in water samples, the *E. coli* bacteria being present among these in between 20 and 100% of the seasonal cases. The species of genus *Pseudomonas* were detected during all seasons, except the spring of 2013.

The increased frequency of bacteriologically positive samples was influenced by the number of rainy days and the amount of precipitation, statistically supported for all studied bacteria types with correlation coefficients between 0.57 and 0.81.

The microbial population isolated in fish is presented in **Figures 4a** and **4b**. There were no statistically significant differences between the total flora isolated from gills and hepatopancreas or the distinctive bacteria, based on feeding habits and habitat (**Tables 1** and **2**).

Antibiograms performed on these isolates indicated that there were changes in overall antibiotic sensitivity and resistance depending on the season (**Figures 5a** and **5b**). The inhibition diameters were variable, depending on the strain from 11 to 26 mm; nevertheless, the average
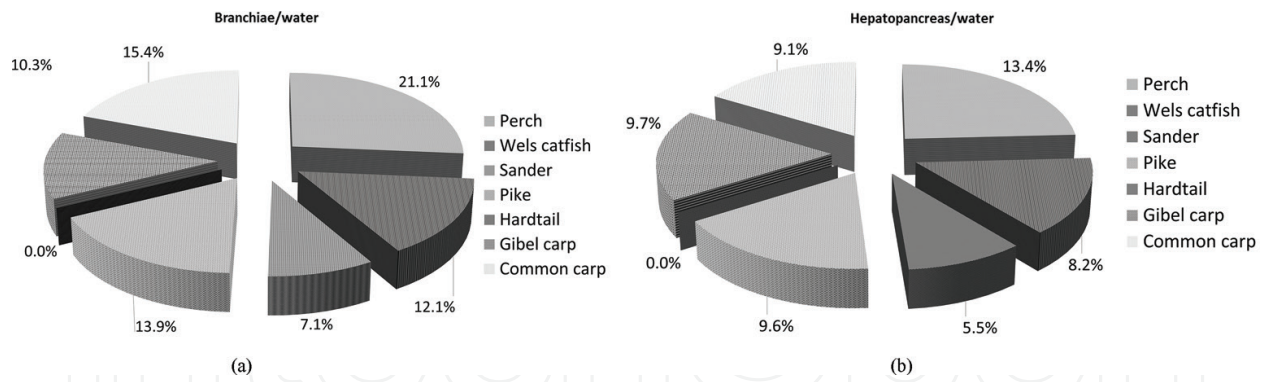
Figure 4. (a) The frequency of bacterial isolates by species, found in gills and also the habitat water. (b) The frequency of bacterial isolates by species, found in hepatopancreas and also the habitat water.

Species	1	2	3	4	5	6	7	Total
Perch	0.05	0.21	0.32	0.63	0.74	0.74	0.47	0.45
Wels catfish	0.44	0.41	0.46	0.28	0.47	0.58	0.18	0.40
Sander	0.30	0.20	0.30	0.20	0.70	0.80	0.20	0.39
Pike	0.44	0.50	0.47	0.35	0.50	0.53	0.24	0.43
Pontic shad	0.20	0.40	0.40	0.30	0.60	0.70	0.40	0.43
Crucian carp	0.29	0.24	0.30	0.23	0.37	0.51	0.19	0.30
Common carp	0.24	0.33	0.39	0.31	0.65	0.82	0.20	0.42

1—*V. cholerae, V. fluvialis*; 2—*V. alginolyticus, V. metschnikovii*; 3—*V. mimicus, V. vulnificus*; 4—*V. parahaemolyticus*; 5—*E. coli*; 6—coliform bacteria; 7—*Pseudomonas* spp.

Table 1. The frequency of bacteria from gills of fish that differ by habitat and feeding habits.

Species	1	2	3	4	5	6	7	Total
Perch	0.05	0.11	0.21	0.42	0.68	0.84	0.63	0.42
Wels catfish	0.31	0.38	0.44	0.33	0.54	0.69	0.26	0.42
Sander	0.30	0.30	0.30	0.10	0.40	0.90	0.40	0.39
Pike	0.38	0.41	0.32	0.29	0.50	0.59	0.33	0.40
Pontic shad	0.50	0.40	0.30	0.50	0.70	0.90	0.40	0.53
Crucian carp	0.23	0.26	0.30	0.26	0.39	0.64	0.36	0.35
Common carp	0.16	0.14	0.31	0.29	0.41	0.76	0.39	0.35

1—*V. cholerae, V. fluvialis*; 2—*V. alginolyticus, V. metschnikovii*; 3—*V. mimicus, V. vulnificus*; 4—*V. parahaemolyticus*; 5—*E. coli*; 6—coliform bacteria; 7—*Pseudomonas* spp.

Table 2. The frequency of bacteria from hepatopancreas of fish that differ by habitat and feeding habits.
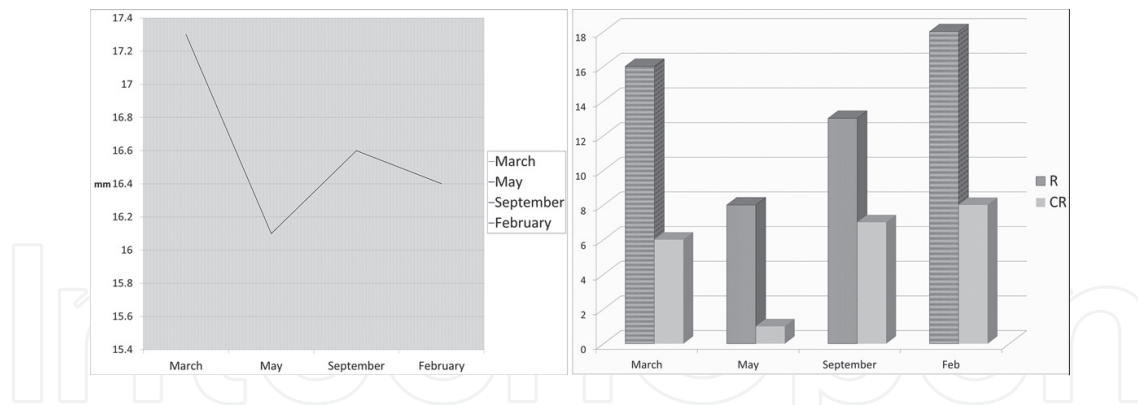
Figure 5. (a) Changes in overall inhibition (average—all species, all antibiotics) diameters (mm) by season. (b) Variation in cumulated total resistance (R) and resistant colony (CR) numbers by season.

diameters calculated by antibiotic type differ significantly for May and February ($p < 0.05$) (14–19 mm in March, 11–21.5 in May, 12–19 in September, and 11.5–20.4 in February).

The highest total resistance was present in March and February, at similar levels, while May, September, and February had the least R and CR. The only antibiotic with no R or CR in any of the seasons was the ciprofloxacin. There were no R and CR to enrofloxacin in February.

The isolated *E. coli*, coliform, and *E. faecalis* strains showed multidrug resistance, to 7 or 8 of the tested antibiotics, at different levels. The presence of resistant colonies stood for the partial inefficacy of the antibiotics used against these strains. This phenomenon is further posing the risk of selective pressure toward MDR in these locations in case of their use and increasing the consumer risk. The results indicated the presence of antibiotic resistance indicator bacteria in the tested samples and also showed that the changes in antibiotic resistance depended on the season, rather than on fish species, their feeding habits, or habitat.

5.3. Conclusions

There were no obvious direct relations observed between the two types of pollution, toxicological and microbial, the only relationship being pointed out after a spatial variation analysis using the geographic information system (GIS) technique, where areas could be ranked by having the lowest or highest pollutant values, and possible input areas.

There were similarities regarding the spatial pattern of the As, Hg, and Pb, together with *Vibrio* species and *E. coli* according to their average water values, having maximum loads in the Sf. Gheorghe branch and its secondary delta. The upstream pollution was the one that characterized the size and intensity of the contaminated areas, with the hydrological conditions influencing the local spatial distribution and the time of retention.

The research regarding the harmful effects of habitat pollution upon the fish population studied in the Danube Delta aimed to examine the bioaccumulation of heavy metals. Studying the most common fish species, it can be stated that arsenic level was predominant, compared to other metals, and by ignoring it for ranking purposes, carp is the first species, due to higher levels of lead and cadmium.

Pontic shad (*Alosa immaculata*) bioaccumulates heavy metals differently, indicating that it had different routes of exposure, being an allochthone species that was not relevant in the eco-toxicological assessment of deltaic habitats. Bioaccumulation of heavy metals was different among species and types of analyzed organs (internal or external), nonpredatory species bio-accumulating mainly Pb and Cd, while predatory species especially accumulating Hg.

In spite of the heavy microbial pollution of the investigated fish, this study could not clearly identify any species to be outstanding in terms of microbial contamination, regardless of samples originating from the gills or hepatopancreas. *E. coli* had the broadest distribution, followed by several species of the *Vibrio* genus.

Several recent studies pointed out the importance of the environmental nonpathogenic microbiome in transferring antibiotic resistance to pathogenic bacteria shed by diseased or convalescent individuals [23]. Since heavy metal and antibiotic resistance are connected [24, 25], tolerance to both of those pollutants is frequent; the higher the level of heavy metal pollution, the more numerous the antibiotic-resistant bacteria [25]. Heavy metal pollution was suggested to exert a selective pressure with both environmental and clinical importance [2]; therefore, in the Danube Delta, the simultaneous presence of zoonotic bacteria in the environment and fish and medium levels of heavy metal pollution in the same areas could contribute to endemic antibiotic resistance and upsurge the risk for both humans and animals.

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